Transfer of conjugative plasmids among bacteria under environmentally relevant conditions

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Preface

This thesis is based on research done for a PhD project undertaken from January 2007 to December 2009 at the Department of Environmental Engineering, Technical University of Denmark. The internal supervisor was Professor Barth F. Smets, Department of Environmental Engineering (DTU), and co-supervisor was Professor Jan Sørensen, Section of Genetics and Microbiology, Faculty of Life Sciences, University of Copenhagen.

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The thesis is composed of a summary of a subject "*Transfer of conjugative plasmids among bacteria under environmentally relevant conditions*" and three journal papers (2 submitted and 1 manuscript).

Papers comprised in the thesis include:

I. **Sanin Musovic**, Arnaud Dechesne, Jan Sørensen & Barth F. Smets.
A novel assay to measure the permissivity of microbial communities towards horizontal receipt of exogenous mobile elements. *Submitted manuscript. Applied and Environmental Microbiology (AEM02713-09).*

II. **Sanin Musovic** and Barth F. Smets. The toxic effect of elevated Hg doses is alleviated by *in situ* transfer of the Hg resistance plasmid, pKris-1, to a synthetic microbial community. *Manuscript.*

III. **Sanin Musovic**, Charles Halouze, Claus Sternberg and Barth F. Smets. The effect of physiological status of Pseudomonas putida KT2442 on transfer rate and proficiency of a conjugal TOL plasmid. *Submitted manuscript. FEMS Microbiology Letters (FEMSLE-10-01-0074).*

The papers are not included in this www-version but can be obtained from the library at DTU Environment. Contact info: Library, Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2800 Kgs. Lyngby, Denmark or library@env.dtu.dk.

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Kgs. Lyngby
January 2010 *Sanin Musovic*

Abstract

Novel analyses of bacterial genome sequences have revealed that extensive exchange of genetic material via a process of horizontal gene transfer (HGT) has occurred among bacteria in the environment. Conjugation, one HGT mechanism mediated by mobile genetic elements (e.g. plasmids), is suggested to play an essential evolutionary role for bacteria and to be crucial for their rapid adaptation to changing environmental conditions. Plasmids frequently carry accessory functions such as antibiotic resistance or catabolic- or xenobiotic degrading genes, increasing their relevance in medical bacteriology, wastewater purification and bioremediation. Although the evidence of the occurrence of HGT and its importance to bacterial evolution are undisputed, these still do not explain the extent of transfer of mobile genetic elements in the environment. Therefore, new studies examining the degree of conjugal gene exchange and its regulation by various environmentally relevant factors are required. As the majority of bacteria in the environment are not culturable on commercial laboratory media, the novel methods and approaches that reduce these biases need to be developed and continuously optimized. Quantifying conjugal gene transfer under environmentally relevant conditions, the fraction of bacteria taking part, and the role of environmental factors ultimately will allow prediction of the behavior of various plasmids in natural environments.

The aim of this PhD thesis was to 1.) quantify the fraction and diversity of a microbial community that can receive and maintain a mobile genetic element, 2.) investigate the extent of plasmid dissemination under environmentally relevant oligotrophic conditions and the presence of sub-toxic selective pressure, and 3.) examine the effects of the plasmid donor cell's physiological status on transfer rates and estimate the fraction of transfer proficient cells.

The spreading of mobile genetic elements to microbial communities has been demonstrated by various studies. However, very little is known about the fraction of a microbial community that can be engaged in plasmid dissemination. In Section 1, I have developed a new approach to, for the first time, quantify the true fraction of a microbial community partaking in plasmid exchange. A cultivation-minimal method in combination with reporter gene technology and advanced microscopy were used in monitoring and quantification of transfer incidences for RP4:*gfp* plasmid from *Pseudomonas putida* to indigenous bacteria

in soil suspension. The RP4 plasmid, isolated from a hospital in Birmingham, belongs to the IncP-1 group of plasmids who carry diverse accessory functions and are frequently found in the environment. An exceptionally high transfer incidence of the RP4:*gfp* plasmid (up to 1 transfer per 2.000 soil bacteria) was observed. In addition, a broad host range among soil bacteria including *alpha, beta* and *gamma* subclasses of *Proteobacteria* suggests a quick spread and persistence of IncP-1 antibiotic resistance plasmids to indigenous soil bacteria, which might constitute a serious public health threat. The new method revealed a wider phylogenetic range of plasmid recipients than observed by traditional selective cultivation-based method.

One of the most widespread hazardous pollutants in the environment, largely due to industrial contamination, is mercury. The recently revealed high abundance of conjugal plasmids at highly mercury-contaminated sites, in combination with the fact that mercury resistance-encoding genes are often located on mobile genetic elements, increases the relevance of studies investigating the contribution of plasmids to bacterial adaptability. Therefore, in Section 2, I examined the dissemination capacity of an IncP-1 plasmid (pKris-1), under environmentally relevant nutrient-poor (oligotrophic) conditions and the presence of mercury selective pressure. The pKris-1 plasmid, isolated from heavily mercury contaminated soil at East Fork Poplar Creek (USA), encodes mercury resistance by the *merA* gene. Using a cultivation-independent quantitative-PCR method, it was demonstrated that plasmid dissemination from *E. coli* donor strain to a synthetic bacterial communities occurred under oligotrophic conditions. In addition, we demonstrated that the plasmid transfer rate was greatly enhanced (up to 1.000 fold) in microcosms continuously exposed to the highest tested mercury dose (1,0 μM), than in microcosms exposed to zero or even 0,1μM Hg^{+}. Only the communities with larger plasmid dissemination were equipped with a "rapid response" feature to an elevated toxic 10 μM Hg^{+} mercury disturbance. The observations increase relevance of IncP-1 plasmids in potential bioaugmentation process at contaminated sites.

Substrate availability has in general been positively linked to increased plasmid transfer among bacteria, but previous studies were, however, unable to differentiate between a true plasmid transfer to a recipient (plasmid-free) cell and a subsequent outgrowth, or ensure a similar physiological status of the majority of plasmid donor cells. In Section 3, I investigated the effect of physiological

status of a TOL (IncP-9) plasmid-carrying *P. putida* KT2442 strain on transfer rates to two *Pseudomonas* recipient strains in nutrient-free liquid conditions. The results showed that different antecedent growth conditions leading to different growth rates, had, within the range examined, very little impact on the plasmid transfer kinetic rates inferred by selective plating or the flow cytometric approach using fluorescent reporter genes (i.e. *gfp* and *dsred*). However, the transfer rate was 1000 times higher when inferred with the latter approach. A surface mating approach constructed to optimize cell-to-cell contact with recipient cells revealed that only a minor fraction of plasmid donor cells were transfer proficient on surface media (ca. 8 percent). The fraction was further reduced (ca. 100 times) in stirred liquid conditions, suggesting a larger dissemination proficiency for TOL plasmid in stagnant versus mixed conditions.

In conclusion, the studies presented in this PhD thesis have contributed to a better understanding of conjugative plasmid transfers among bacteria by developing a novel tool to quantify the fraction of an indigenous community partaking in plasmid transfer, revealing the extent of plasmid transfer under oligotrophic (nutrient poor) conditions, and the effects of donor physiological status or advantageous selective pressure on plasmid dissemination.

Dansk sammenfatning

Dansk titel: Overførsel af konjugerbare plasmider mellem bakterier under de miljørelevante forhold

De seneste analyser af bakterielle genomer har vist at en solid udveksling af genetisk materiale via processen horisontalt gen overførsel (HGO) har fundet sted mellem bakterier i miljøet. Konjugation, en af HGO mekanismer der medieres af mobile genetiske elementer (f.eks. plasmider) antages at have en meget vigtigt evolutionær rolle for bakterier, og at den er meget betydningsfuld for deres hurtige tilpasning til ændringer i miljøet. Plasmider bærer ofte ekstra funktioner såsom antibiotikaresistens, kataboliske- og xenobiotiske nedbrydnings gener, der øger deres relevans for medicinal bakteriologi, oprensning af forurenet vand og bioremediering. Selvom beviserne på HGO og dens betydning for den bakterielle evolution er ubestridte, så forklarer disse ikke graden af overførsel af mobile genetiske elementer i miljøet. Derfor er nye studier der undersøger graden af den konjugative gen udveksling og dens regulering via diverse miljørelevante faktorer nødvendig. Da de fleste bakterier i miljøet ikke er dyrkbare på de kommercielle laboratoriske media, bør nye metoder og tilgange der reducerer disse mangler udvikles og kontinuert optimeres. Kvantificering af den konjugative gen overførsel under de miljørelevante forhold, den del af bakterierne der er involveret, og rollen de miljørelevante faktorer har på konjugation, vil muliggøre den ultimative forudsigelse af plasmidernes adfærdsmønster i naturen.

Formålet med denne PhD-afhandling var at 1.) kvantificere den fraktion og diversitet af et mikrobiel samfund der er i stand til at modtage et mobil genetisk element, 2.) undersøge omfanget af plasmid spredning under de miljørelevante (substrat-begrænsede) oligotrophiske forhold og tilstedeværelse af sub-toksisk selektiv tryk, og 3.) undersøge effekten af plasmid donor cellens fysiologiske status på overførselsraten, samt vurdere fraktionen af overførsels-effektive celler.

Spredning af mobile genetiske elementer i mikrobielle samfund er blevet demonstreret i forskellige studier. Alligevel, er der meget lidt kendskab til den

del af det mikrobielle samfund, der kan involveres i plasmid spredning. I Sektion 1, har jeg udviklet en ny metode til at kvantificere fraktion af det mikrobielle samfund der kan modtage og opretholde et konjugativ plasmid. Dyrknings-minimal metode i kombination med reporter gen teknologi og moderne mikroskopi blev brugt til at overvåge og kvantificere tilfælde af overførsler for et RP4:*gfp* plasmid fra *Pseudomonas putida* til oprindelige jord bakterier i celle suspension. RP4 plasmidet, der er fundet på et hospital i Birmingham, hører til i IncP-1 gruppen af plasmider, hvis medlemmer bærer forskellige ekstra funktioner og er et hyppigt fund i miljøet.
Den meget høje rate af RP4:*gfp* plasmid overførsel (op til 1 overførsel per 2.000 jord bakterier) blev vist. Desuden, det brede værtskab mellem jord bakterier inklusive *alpha, beta* og *gamma* sub-klasser af *Proteobacteria* indikerer en hurtigt spredning og opretholdelse af IncP-1 antibiotika resistente plasmider blandt jord bakterier, og kan udgøre en seriøs trussel på menneskers helbred.

Et af de mest spredte og farefulde forureningsstoffer i miljøet, primært som følge af den industrielle forurening, er kviksølv. For nylig er der påvist en større mængde af konjugative plasmider ved kviksølv forurenede områder. I kombination med det faktum at kviksølv resistente gener ofte er båret af mobile genetiske elementer, øger relevansen for studier der undersøger bidraget af plasmider til den bakterielle adaptationsevne. Derfor, i Sektion 2, undersøger jeg spredningskapaciten af et IncP-1 plasmid (pKris-1), under de miljørelevante næringsstof-begrænsede (oligotrophic) forhold og med tilstedeværelse af kviksølv selektive tryk. pKris-1 plasmid, isoleret fra en meget tungt forurenet jord ved East Fork Poplar Creek (USA), koder for kviksølv resistens via *merA* genet. Ved en dyrknings-uafhængig kvantitativ-PCR methode blev det demonstreret at plasmid spredning fra *E. coli* donor stamme til et syntetisk bakterielt samfund finder sted under oligotrophic forhold. Desuden, demonterede vi at plasmid overførsels raten var meget forhøjet (op til 1.000 gange) i mikrokosmos kontinuert udsat for den højest testede kviksølv dosis (1,0 μM) i forhold til mikrokosmos udsat for nul eller 0,1 μM Hg^{+}. Kun de samfund med store plasmid spredning var efterfølgende udstyret med en ”hurtig respons” egenskab mod en øget og toksisk 10 μM Hg^{+} kviksølv. Disse resultater øger

relevansen af IncP-1 plasmider i en potential bioaugmentation proces ved forurenede grunde.

Substrat tilgængelighed er normalt generelt positivt linket med en øget plasmid overførsel imellem bakterier, men de tidligere studier var ikke i stand til at skelne imellem den rigtige plasmid overførsel til en recipient (plasmid-fri) celle og efterfølgende vækst, eller at sikre en lignende fysiologiske status af de fleste plasmid donor celler. I Sektion 3, undersøgte jeg effekten af den fysiologisk status af et TOL (IncP-9) plasmid bærende *P. putida* KT2442 stamme på overførselsrate til to *Pseudomonas sp.* recipiente stammer i et substrat-fri fluidum–forhold. Resultaterne viste at de forskellige vækstforhold, der resulterede i forskellige vækstrater, i det undersøgte område, havde meget lidt effekt på plasmid kinetiske overførselsrater, beregnet ved den dyrkningsbaserede selektive metode eller flow cytometric metode med de fluorescerende reporter gener (i.e. *gfp* and *dsred*). Transfer raten var dog 1.000 gange højere ved den sidstnævnte metode. En overflade-mating metode konstrueret til at optimere celle-til-celle kontakt med recipient celler viste at kun en lille fraktion af plasmid donor celler var i stand til at overføre plasmidet (ca. 8 procent). Fraktionen var yderligere reduceret (ca. 100 gange) i rystende-væske forhold, der indikerer et større sprednings potentiale for TOL plasmid i stillestående end i rystende forhold.

I konklusion. studierne præsenteret i denne PhD afhandling har bidraget til en bedre forståelse af konjugative plasmid overførsler mellem bakterier ved at udvikle et nyt redskab til at kvantificere den fraktion af det oprindelige bakteriesamfund der tager andel i plasmid overførsel, afsløring af størrelsen af plasmid overførsel under de oligotrophic (substrat-begrænsede) forhold, og effekten af donor fysiologiske status eller det fordelagtige selektive tryk på plasmid spredning.

1. Introduction

The novel studies of whole bacterial genomes have revealed that bacteria have through the evolutionary process exchanged a significant part of their genetic material via a process of horizontal gene transfer (HGT) (Jain et al., 1999; Gogarten and Townsend, 2005). Recent genomic studies on *Escherichia coli* have revealed that more than 24% of its genome has been acquired from other bacteria by HGT (Lawrence and Ochman, 2002). The newly acquired genetic material might, however, carry genes encoding certain environmentally beneficial (e.g. pollutant degradation) or human health relevant (e.g. antibiotic resistance) traits.

One of the historically greatest achievements of medicine was the discovery of antimicrobial drugs capable of controlling bacterial infectious diseases. However, just a few years after the first mass-production and application of pioneering antibiotics (e.g. penicillin) in the early 1940, researchers reported the first larger incidences of antibiotic-resistant bacteria. Since then, an increasing application of antibiotics in agriculture, veterinary- and human medicine, was followed with an increasing number of reported antibiotic-resistances among pathogenic, opportunistic or normal flora bacteria flora from man, animals, food and the environment (Datta, 1969; Roberts, 1996). Emergence of resistance genes in environmental bacteria to clinical antibiotics is not surprising, as the billion-years-long evolution of bacteria and their coexistence with naturally antibiotic-producing microbiota have provided them with a large degree of genetic diversity, hence resistant bacteria might occasionally be isolated from apparently nonselective environments (Mach and Grimes, 1982).

A large antibiotic usage (e.g. 25 mil. kg per year in the United States) is alarming, in view of the fact that a major fraction of the antibiotics used as growth promoters in food production (ca. 90%) is excreted from treated animals (pers. communication Holger H.), while ca. 40% of antibiotics used in human medicine also are excreted to the environment (Boxall et al., 2004; Kummerer, 2004). Although the European Union has gradually reduced the use of growth-promoting antimicrobials, these are still massively used as therapeutic agents in food-producing animals (e.g. 400 tones per year in United Kingdom) (Guardabassi and Courvalin, 2006; Enne et al., 2008)

A number of studies have directly linked the application of antibiotics with a massive occurrence of e.g. tetracycline, vancomycin or streptothricin resistant bacteria in the intestines of treated humans, pigs and broilers (Tschape et al., 1984; Hummel et al., 1986; Aarestrup et al., 2000). Therefore, seasonal application of sewage sludge and manure as soil fertilizers can introduce large amounts of active antibiotics and antibiotic-resistant intestinal bacteria into the soil. The exposure of environmental organisms to antimicrobial agents and resistant species might create a reservoir of resistance traits among indigenous bacteria, which can have broader consequences to public health.

A rapid increase in antibiotic resistant bacteria is often suggested to be the result of horizontal acquisition of new resistance genes carried by mobile genetic elements (e.g. plasmids). However, mobile genetic elements were also shown to carry certain beneficial catabolic genes involved in degradation of a variety of polluting compounds (Summers and Lewis, 1973; Don and Pemberton, 1981; Top et al., 1998a).

These observations increase the relevance of studying the occurrence and extent of horizontal gene exchange among bacterial communities, as that could be one of the greatest threats or beneficial tools in the modern world. Therefore, through this PhD thesis I will review the relevant literature on horizontal gene transfer, with a focus on a rapid gene exchange via one transfer mechanism, i.e. conjugation, its extent and effects of environmental controlling factors.

2. Bacterial genetic modification

Asexual bacterial replication ensures identical offspring and spread of the inherited genes to the next generation. However, bacteria can periodically accomplish modification of their genetic information (i.e. DNA) by: 1. accumulation of spontaneous mutations or inter-genomic homologous recombination with a foreign chromosomal DNA fragment nearly identical in nucleotide sequence (Milkman et al., 1999; Dutta and Pan, 2002) and 2. introduction of a completely new set of genes by horizontal gene transfer (HGT) (Lawrence, 1999). The latter process might rapidly provide bacteria with a new set of genes in comparison to slow modification of existing genes by accumulation of functional point mutations (for review see Majewski, 2001). The importance of horizontal gene transfer in bacterial evolution has been suggested by numerous studies. The presence of a particular phenotypic trait, e.g. tetracycline-resistance-conferring *tetM* gene or a particular operon (a cluster of genes) among diverse bacterial lineages, is suggested to be the result of horizontal gene transfer (*for review see* Davison, 1999; Koonin et al., 2001), although a theoretical possibility of their independent emergence might not be excluded (Ochman et al., 2000).

2.1 Mechanisms of horizontal gene transfer

Horizontal gene transfer (HGT) among prokaryotic organisms consists of three recognized mechanisms: transduction, transformation and conjugation.

Transduction, which is transfer mediated by bacterial viruses (bacteriophages) is suggested to have only a slight contribution to bacterial gene exchange due to extreme bacteriophage sensitivity (e.g. temperature, pH) and narrow bacterial specificity (Bergh et al., 1989; Dröge et al., 1999).

Transformation, a process of uptake and chromosomal integration of exogenous DNA was revealed under optimal *"in vitro"* conditions among ca. 40 different bacterial species (genera of *Bacillus*, *Streptococcus*, *Azotobacter*, *Acinetobacter*, *Vibrio*, *Pseudomonas etc*) (*for review see* Lorenz and Wackernagel, 1994). Although the transformation has been demonstrated in a variety of natural ecosystems, e.g. soil and marine water and sediment microcosms, (Stewart and

Sinigalliano, 1991; Nielsen et al., 1997), the true extent of transformation under environmental conditions is still poorly understood. However, it was suggested that, due to e.g. absence of DNA specificity in several transformation systems, transformation could have a noteworthy contribution in bacterial evolution.

The conjugative mechanism relies on exchange of mobile genetic elements (e.g. plasmids, conjugative transposons). Conjugation is believed to be the most important mechanism responsible for short-term bacterial adaptation (Sorensen et al., 2005), transferring genetic material even between phylogenetically-remote organisms (Lilley et al., 1994; Dröge et al., 1999); hence it could also significantly contribute to bacterial evolution. Conjugation will be the main subject of the present thesis.

3. Horizontal gene pool: Mobile Genetic Elements

Horizontal gene exchange by MGEs was suggested to be essential for bacterial adaptation and successful colonization of new ecological niches (Top et al., 2000). A large number of chromosomal- or extrachromosomal mobile genetic elements such as plasmids, IS elements, integrons, genomic islands, gene cassettes, transposons, and conjugative transposons constitute a horizontal gene pool (Hacker and Carniel, 2001; Toussaint and Merlin, 2002). The definition of MGEs should also include bacteriophages (Thomas, 2000), although these will not be further discussed here.

3.1 Conjugative transposons and plasmids

The conjugative transposable genetic elements (transposons) such as Tn*916,* Tn*918*, Tn*5030* can promote, via their transposition functions encoded by excisase and integrase genes, their intracellular transposition or transfer as single-stranded DNA (ssDNA) to a recipient cell via a cell-to-cell contact (Rice, 1998). As conjugative transposons frequently carry antibiotic resistance genes, e.g. *tetQ* on Tn*5030* (Salyers et al., 1995) *or tetM* gene on Tn*916* (Showsh and Andrews, 1992), these are of great importance when considering human health threats. Moreover, Tn*916* was shown to transfer even among phylogenetically distant (i.e. Gram-positive and Gram-negative) bacteria (Bertram et al., 1991; Clewell et al., 1995).

Plasmids are by definition extrachromosomal fragments of DNA, which replicate autonomously from the chromosomal DNA, although their replication process is typically synchronized with the metabolic state of the host bacterium (for review see Couturier et al., 1988). Plasmids normally have mosaic-like structures, as they are normally composed of series of genetic sequences (e.g. integrons, transposons, IS elements etc) on a plasmid backbone (Figure 1, a model of pB10 plasmid), which might provide bacteria with a selective advantage in exchange for stable maintenance (Boyd et al., 1996; Osborn et al., 2000).

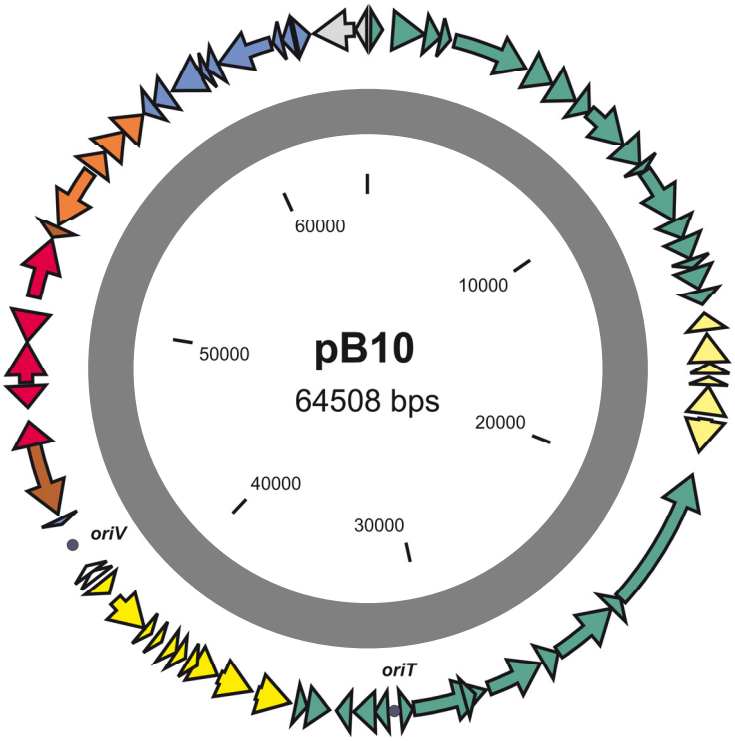

Figure 1. Genetic map and mosaic structure of an IncP-1β plasmid pB10. Coding regions are shown by arrows. The functional modules of the plasmid backbone: mpf trb-genes (green, up), transfer tra-genes (green, down), regulative and stable inheritance genes (yellow); replication ssb–trfA1 genes (grey), class 1 integron (light yellow), Tc^r (tetracycline) resistance transposon (red), Tn501-like mer^r transposon (dark blue), Sm^r transposon (orange), IS1071 element (brown), , and genes of unknown function (white). The origins of vegetative (*oriV*) and transfer (*oriT*) replication (black circles) *Modified from* (Schluter et al., 2003).

Plasmids vary in size and structure, but the absolute minimum that ensures plasmid persistence among bacterial community is the *origin of replication* (*OriV*) (Fig. 1), a site crucial for initiation of plasmid replication, located in a *replicon* region, which in the case of large plasmids might occur more than once on a plasmid (Thomas, 1981). The replicon region, beside the *OriV*, generally consists of genes involved in the initiation and control of replication (e.g. *co*p, *inc*, *rep*) (*see below*).

The conjugal gene transfer in the environment, according to the existing paradigm, occurs exclusively between closely related bacteria, e.g. members of Gram-negative or Gram-positive bacteria, although exchange of mobile genetic

elements has been reported among highly divergent bacteria (i.e. Gram-positive and Gram-negative) (Bertram et al., 1991) or even with archea, plants and yeast (interkingdom) (for review see Koonin et al., 2001). Conjugative gene exchange among Gram-positive bacteria in the environment is very relevant, however in this thesis, due to a significant difference in the conjugative mechanisms compared to Gram-negative bacteria (e.g. interaction via the release of specific clumping factors (adhesion molecules) by plasmid donor bacteria in response to pheromones from recipient cells (Clewell et al., 2002; Grohmann et al., 2003), rather than via pilus-like structures), we will through the thesis focus only on conjugation mechanisms among Gram-negative bacteria, as the plasmids studied belong to this group of bacteria.

4. Plasmid transfer systems

The first published research on conjugal exchange of circular DNA elements by a sex-resembling process among intestinal *E. coli* bacteria was reported in 1946 by Joshua Lederberg and Edward Tatum at Yale University (USA) (Tatum and Lederberg, 1947). Since then, the conjugative systems have been emphasized, mainly due to importance of spreading of antibiotic resistance genes via conjugative plasmids.

Self-transmissible conjugal plasmids carry the genes necessary for transfer initiation at origin of transfer (*OriT*) and mating-pair apparatus formation (Zatyka and Thomas, 1998). However, a large group of plasmids frequently lack genes for the mating pair apparatus, but might be co-transferred (non self-transmissible but mobilizable) via a mating apparatus provided by a self-transmissible plasmid, as they also contain their own *ori*T and genes for conjugal DNA processing.

Historically, the majority of molecular and biochemical understanding of the conjugative processes was derived from the examination of F plasmid conjugation system in *Escherichia coli* (Manchak et al., 2002), today recognized as the F-like conjugation system (IncF I, II, III and IV group) (Frost et al., 1985; Finlay et al., 1986; Manwaring et al., 1999). Other plasmids studied in detail are RP4, R388, Ti-plasmids etc. (Pansegrau and Lanka, 1996). Although these conjugative plasmids differ significantly in the organization of transfer genes, pilus type, etc., they still share certain similarities to IncF plasmids (Llosa et al., 2002). To investigate the particular role of a gene in conjugation processes, researchers have often used experiments involving knockout-mutations of a functional gene (Grahn et al., 1997).

As the two most relevant plasmids in the current thesis (i.e. RP4 and pKris-1 plasmids) belong to an incompatibility IncP-1 group (plasmid classification into incompatibility Inc groups is described on page 17), the IncP-1 conjugative system is reviewed in the next section and the core similarities and differences to a third relevant TOL (incompatibility IncP-9 group) plasmid will be listed.

4.1 Conjugative system of IncP-1 plasmids.

The conjugative system of the RP4 (IncP-1) plasmid (60 kb) is one of the most intensively studied transfer system at the moment (Samuels et al., 2000). Due to a high genetic homology revealed by numerous of comparative experiments among IncP-1 plasmids (Adamczyk and Jagura-Burdzy, 2003), it is assumed that the conjugative system of RP4 plasmid is representative of other plasmids of the IncP-1 group.

The RP4 plasmid (a.k.a. RK2, RP1, a Birmingham group of indistinguishable plasmids) (Thomas and Smith, 1987; Pansegrau et al., 1994; Zatyka et al., 1994) have a transfer system divided in two regions: Tra1 (*tra*-genes, 13kb region) and Tra2-(*trb* genes, 11,2kb region), which are separated by a 10 kbp region containing transposons and housekeeping genes (Figure 2) (Haase et al., 1995; Waters, 1999).

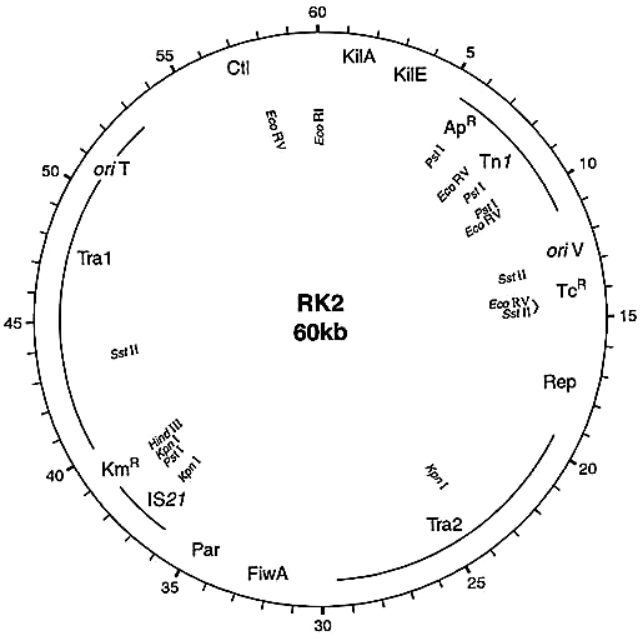

Figure 2. Map of the 60 kb IncP conjugative RK2 (a.k.a. RP4) plasmid. The conjugative transfer genes are clustered in two regions, Tra1 and Tra2. Other genetic markers: IS elements; Tn transposon; Ap (ampiciline) Km (kanamycin), Tc (tetracycline) resistance genes; the origins of vegetative (oriV) and transfer (oriT) replication. The mapping completed by Waters V. L. (Waters, 1999) according to the abbreviation conventions established by (Pansegrau et al., 1994).

In general, genes of the Tra1 region encode proteins for DNA replication and transfer, while genes of the Tra2 region encode the majority of proteins essential for the formation of the mating-pair complex (Mpf), as well as one transfer (*TraF*) protein (Samuels et al., 2000). Coordinated activities of both regions are required for successful plasmid transfer.

The Tra1 region of the RP4 plasmid contains 13 genes, where only six genes (*tra* F, G, H, I, J, and K) are found to be essential for conjugal transfer (Furste et al., 1989; Cabezon et al., 1997)**.** The conjugative transfer of IncP-1 plasmids starts with initial binding of TraJ to a 10-bp *srj* sequence upstream of the *nic* site located within the origin of transfer *oriT* (Fig.2) (Pansegrau and Lanka, 1996). TraI relaxase binds to an *sri* sequence and initiates hydrolysis of a phosphodiester bond at a cleavage-specific *nic*-site of *ori*T (Pansegrau et al., 1990a). Additional binding of Tra1 region encoded TraH and TraK proteins, which have, respectively, a complex-stabilizing and a still unknown function, form a relaxosome complex (TraH, TraK, Tra I,Tra J, *srj**DNA) (Pansegrau et al., 1990b; Ziegelin et al., 1992). The TraI relaxase mediated cleavage of a single phosphodiester bond at *OriT* (Grohmann et al., 2003). The 5′ terminus of cleaved ssDNA (T-strand) is transferred to a recipient bacterium in the 5′- 3′ direction, and replaced by a DNA polymerize (III) mediated replication in the donor cell.

Tra2 region`s *trb* genes are in combination with a TraF pilin-processing protease, responsible for mating-pair formation (Mpf), i.e. assembly of pili, formation of transport channels (pore) and cell aggregation (Haase et al., 1995; Grahn et al., 1997; Bates et al., 1998). TraF protease plays a crucial role in maturation of the *trbC* gene encoded pilin-precursor (Eisenbrandt et al., 2000). The importance of RP4 pili (thin and rigid nature) in the formation of a cell-to-cell aggregate has been confirmed by numerous studies although the role of pili in transfer of a T-strand is still unresolved. Samuels et al. (2000) observed that the RP4 *trbC*¯ plasmid results in pili-deficient donor cells, which, however, still formed conjugative junctions (i.e. region of outer membrane where DNA exchange occurs thought transport channels). Researchers suggested that a larger irregularity among *trbC* mutant (RP4 *trbC*¯) junctions was the reason for a large reduction in transfer frequencies (Haase et al., 1995), as it also might have a role in mating pair stabilization (Samuels et al., 2000). The importance of *trbC* gene on plasmid host range was reported by De Gelder et al. (2008).

Transport of T-DNA starts at the same time as relaxosome-complex binds to a transport pore by a multimeric transmembrane TraG protein, which also assists in T-DNA threading through the pore (Grohmann et al., 2003). Co-transferred DNA primase induces in the recipient cell a quick synthesis of primers required for the generation of a complementary strand by DNA polymerase (Lanka and Wilkins, 1995). Co-transfer of primase and the quick expression of a few "early" genes in the recipient cell ensure DNA replication and establishment of incoming plasmid. The termination of conjugation in the recipient cell ends with a cleavage of the 3′-OH terminus from the intermediate (Pansegrau and Lanka, 1996), and covalent rejoining of T-strand ends.

4.2 Conjugative system of pWWO (TOL) plasmid

pWWO (a.k.a. the archetype TOL) plasmid is a 117 kb self-transmissible plasmid, having a region of 18 kb encoding for all proteins involved in conjugative transfer (Williams and Murray, 1974; Greated et al., 2002). A recent analysis of the pWWO (TOL) plasmid using an open reading frame method for locating genes encoding proteins has suggested that open reading frames (ORF's) are organized in three operons; *traD, traA-C* and the *mpf* operon (Figure 3) (Lambertsen et al., 2004).

Interestingly, the promoters direct the expression of *traA*, *B*, and *traC* genes in one direction, while expression of *traD* gene is regulated in the other direction. These two operons encode proteins predicted to be involved in relaxasome formation and DNA processing. TraA, TraB, and TraC proteins have shown similarity to Trw proteins sequences of R388, while *traD* have shown sequence homology and positioning to the oriT-region like the *traK* gene of RP4 plasmid (Greated et al., 2002). The *mpf* operon contains all genes required for mating pair formation, and these have shown similarity to proteins of other type-IV secretion system proteins, like the *trb* genes (Tra2 region) of RP4 plasmid (Greated et al., 2002).

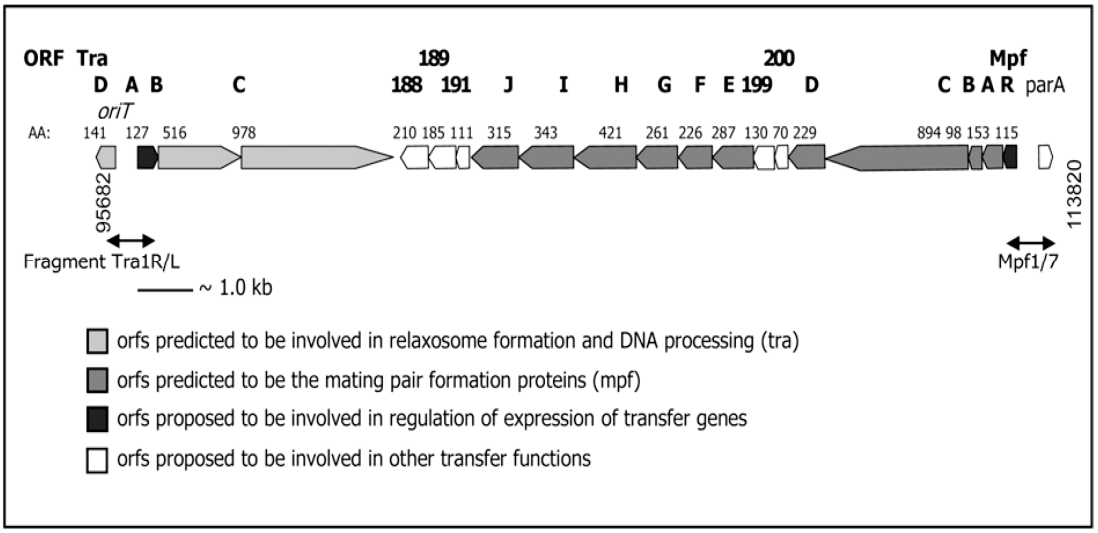

Figure 3. Map of the putative transfer region of pWW0. The encoded ORFs are shown as box arrows, with the pointed end indicating the direction of transcription. The sizes of the predicted polypeptide products are shown above each ORF, and the name of the product is indicated above that. *Tra*; transfer genes and *mpf;* mating pair formation regions. *OriT;* origin of transfer *(Lambertsen et al., 2004)*.

5. Regulation of plasmid transfer and replication systems

5.1 Regulation of transfer gene expression

The expression of the IncP1 plasmid's transfer genes is regulated by two global proteins, KorA and KorB, encoded by a central control region (*ccr*) on the plasmid. Briefly, a direct repression of the IncP1 transfer genes is mediated by KorB and a small *trbA*-gene encoded regulatory protein (TrbA) having *trbB* promoter as a target site (Zatyka and Thomas, 1998) (Figure 4). KorA, however, indirectly participates in down-regulation of *trb* genes expression via induction of the *trbA* gene. The regulatory processes differ for other plasmids, e.g. expression of transfer genes by IncF plasmids is up-regulated by the TraJ regulator, which is normally repressed by FinP and FinO proteins (Koraimann et al., 1996). Up-and-down regulation of the transfer gene system reduces cell metabolic burden and the possibility of an attack by pilus-specific bacteriophages (Kostelidou et al., 1999). The sequence analysis of pWWO (TOL) plasmid has revealed eight genes encoding putative regulatory genes, including regulators showing similarities to KorA (Greated et al., 2002).

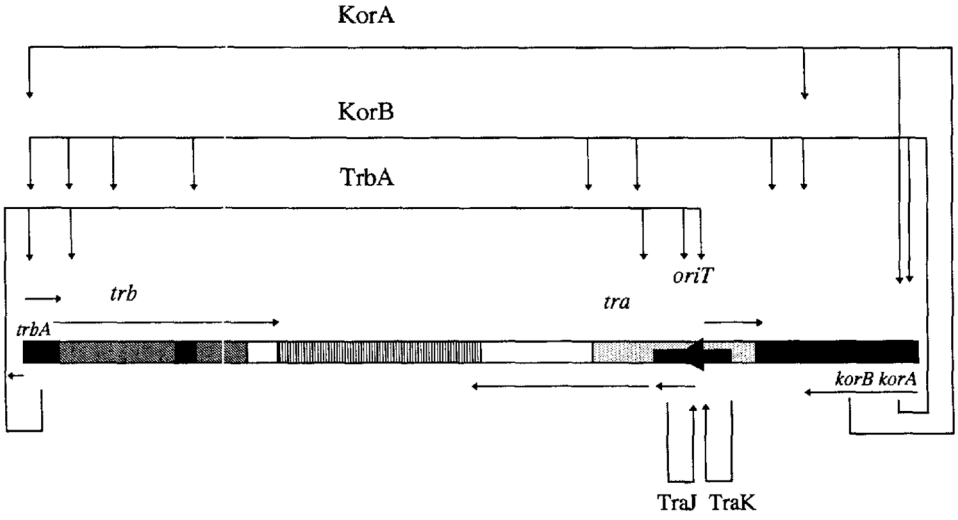

Figure 4. Control of IncP transfer genes. The major control is supplied by three global regulators. KorB and TrbA repress expression of transfer genes directly. KorA derepress *trbA* expression, providing a way of shutting down transcription of the *tra* and *trb* genes. (Zatyka and Thomas, 1998).

5.2 Regulation of plasmid replication system

Tight regulation of the expression of plasmid transfer genes is not the only plasmid self-regulation process. Plasmids, frequently called selfish genetic elements, have evolved copy-number control systems and diverse maintenance functions in order to reduce metabolic burden to a host cell but also ensure their own stable persistence.

The control of plasmid replication in Gram-negative bacteria is largely regulated by an antisense-RNA-mediated control (del Solar et al., 1995; del Solar et al., 1998). The unstable structure of antisense synthesis RNA's will at any point reflect the exact plasmid copy number in a cell. It starts inhibition of the plasmid replication system via regulatory changes of replication initiator proteins or replication primer, the latter only valid for ColE1 plasmids lacking replication initiator proteins. The replication regulatory control mechanism in the case of the R1 plasmid of *E. coli* is mediated by an antisense RNA**,** CopA. The CopA indirectly inhibits the synthesis of a replication-initiator protein RepA, required for initiation of DNA replication at the origin of replication (Blomberg et al., 1994; Malmgren et al., 1997). On average, ca. 50 molecules of RepA protein (i.e. TrfA protein for RP4 plasmid) are required for initiation at the origin of replication (Giraldo and Diaz, 1992). In case of RP4 plasmid, the *trfA* gene is located in the replicon region and encodes a replication initiation protein which interacts with the origin of vegetative replication (*OriV*), thereby regulating the plasmid copy number in a cell (Thomas and Helinski., 1989). The complete regulatory mechanism of RP4 plasmid is still quite unknown, however, it was reported that the expression of the *trfA* gene is well controlled by the *ssb* gene, located upstream of *trfA* (Thomas and Helinski., 1989).

Interestingly, the above described KorB protein-required control of plasmid transfer genes can, along with an IncC protein, play a crucial role in plasmid maintenance. Plasmids, in particular low-copy plasmids, can be lost from a host cell due to segregation of plasmid copies during the cell division process. KorB protein has a crucial centromere-binding role in an active partitioning mechanism (Jacob and Brenner, 1963; Thomas, 1986), reducing the plasmid lose by segregation.

6. Plasmid isolation and classification

The plasmids encoding for certain phenotypes, e.g. antibiotic or heavy metal resistance, are often isolated from various environments by a direct endogenous (Sobecky et al., 1997) or exogenous isolation method (van Elsas et al., 1998; Smalla et al., 2000b; Heuer et al., 2002) in order to reduce cultivation-dependence of indigenous plasmid harboring bacteria. These approaches still rely on the expression of resistance to antibiotics, heavy metals or other selectable traits and will not detect a large range of "cryptic" plasmids whose functions are unknown.

Furthermore these approaches allow researchers mainly to isolate only those plasmids with wanted phenotypes. Interestingly, a large number of studies of several exogenously isolated plasmids (e.g. RP4, pJP4 etc.) have shown transfer mainly among *Pseudomonas sp.* (DiGiovanni et al., 1996; Daane et al., 1996; Top et al., 1998a), suggesting a huge limitation to our current plasmid isolation methods, and a large still-undiscovered pool of diverse "non *Pseudomonas" or "Escherichia coli*" plasmids. Therefore, development of new methods to investigate the diversity and distribution of natural plasmids is required.

6.1 Plasmid classification by incompatibility

Isolated plasmids have been classified according to their physical and phenotypic characteristics, or by incompatibility testing, replicon typing and complete plasmid sequencing (Smalla et al., 2000a). At present, plasmids are mainly classified according to their universal inheritable property, the incompatibility (Inc), which indicate relatedness in their replication system and partitioning (Datta and Hedges, 1972; Novick, 1987), and hinders plasmid coexistence with an "incompatible" group in a cell, but not coexistence of plasmids from different Inc groups.

Plasmid classification based on incompatibility was developed in the early 1970s by Datta and Hedges and has been used to classify many plasmids from Gram-positive and Gram-negative bacteria (Datta, 1979; Couturier et al., 1988). Incompatibility testing is usually accomplished by investigation of the ability of an incoming "unknown" plasmid, introduced by conjugation or a reciprocal transformation experiment (Tietze, 1998), to establish in a cell already containing

another known plasmid. If the resident plasmid is eliminated in the progeny selected for incoming plasmid markers, the incoming plasmid is assigned to the same Inc group (Datta and Hedges, 1971). Although this approach is useful for plasmid classification, the incompatibility testing might be time consuming, due to restricted availability of plasmid marker genes and cell surface exclusion incidents.

In an attempt to improve plasmid classification, scientists have identified unique DNA sequences corresponding to the origins of replication and incompatibility loci of numerous well-characterized plasmids (Couturier et al., 1988). Based on this work, a series of probes and primers specific to replicons from different incompatibility (Inc) groups have been developed (Götz et al., 1996). Currently, a large number of newly-isolated plasmids are quickly classified by the *replicon typing* method, i.e. Southern hybridization using some of the 19 available incompatible Inc-specific replicon *(inc/rep*) probes, representing 17 Inc groups (Elumalai and Mahadevan, 1997; Cook et al., 2001). Although *replicon typing* is a technically simple and fast method, and more reliable for plasmids containing more than one replicon (e.g. IncF plasmids) (Lane and Gardner, 1979; Saadi et al., 1984), it is still biased by a lack of specific *inc/rep* probes for e.g. isolated plasmids from incompatibility groups IncD, IncHI3, IncHII, IncJ, IncV and IncA/C (27, 148, (Couturier et al., 1988; Kobayashi and Bailey, 1994; Sobecky et al., 1997).

PCR amplification of specific plasmid regions (e.g. *oriT* for IncW and IncP; *oriV* for IncQ etc.) (Götz et al., 1996) is also frequently used as a classification method among isolated plasmids, or for monitoring their distribution in the environment (Götz et al., 1996; Smalla et al., 2000b; Smalla et al., 2006; Binh et al., 2008; Malik et al., 2008). For instance, the presence and diversity of IncP-1 plasmids in a wastewater treatment plant was studied by PCR amplification of the *trfA* gene, located in the replicon region of IncP-1 (e.g. RP4) plasmids (Bahl et al., 2009). PCR amplification of the *trfA* gene was also used by Sørensen et al. (Oregaard and Sorensen, 2007) for classification of pKris-1 (IncP-1) plasmid, used in the present study. PCR-based detection of plasmids belonging to IncP, IncN, IncW and IncQ plasmid groups (Götz et al., 1996) is recently extended to cover most plasmid incompatibility groups circulating among the *Enterobacteriaceae*, as the specific primers recognizing FIA, FIB, FIC, HI1, HI2,

I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons have been created (Carattoli et al., 2005).

6.2 Incompatibility IncP-1 and IncP-9 groups

Plasmids isolated in *Pseudomonas* are currently organized in fourteen *Inc* groups, i.e. IncP-1 to IncP-14. A well-studied group of promiscuous plasmids of IncP-1 plasmids are represented with RP4 and pKris-1 plasmids in the current thesis, while the IncP-9 group is represented by a pWWO (TOL) plasmid. It is important to note that some plasmids are, due to their broad host range, frequently classified in more that one Inc group. An example is classification of RP4 plasmid in the IncP-1 group in *Pseudomonas* and into IncP group in *Enterobacteriaceae*, while some plasmids of *Pseudomonas* IncP-4 group (e.g. RSF1010) belong also to the IncQ group in *Enterobacteriaceae.*

The plasmids of the IncP-1 group are regularly associated with antibiotic-, xenobiotic- or heavy metal-resistance markers. Initially, analyses of IncP-1 plasmids by Southern blotting of *HaeII* restriction fragments using the *oriT* region of RK2 as a probe revealed subdivision of the plasmids into *alpha* and *beta* subgroups (Yakobson and Guiney, 1983; Macartney et al., 1997; Vedler et al., 2004). To date, more than 20 IncP-1 plasmids identified from various habitats are completely sequenced and categorized into five (*alpha, beta, gamma, delta* and *epsilon*) subgroups (Boronin, 1992; Schluter et al., 2007).

Plasmids of the IncP-9 group are best known as the vehicles for spreading of biodegradation functions among *Pseudomonas* species, although they also may carry antibiotic resistance determinants. The pWWO (a.k.a. TOL) plasmid is one of the best-studied IncP-9 plasmid found in *P. putida* mt-2 (Williams and Murray, 1974). Plasmid pWWO, along with e.g. pWW53 (Keil et al., 1985) and pDK1 (Kunz and Chapman, 1981), belongs to the TOL (toluene-degradative) plasmid group encoding degradation of the aromatic compounds toluene and xylene (Ramos et al., 1997). These degradation genes are located on two transposons, i.e. Tn4651 and Tn4653. Recently, three completely-sequenced IncP-9 plasmids, pWW0 (Greated et al., 2002), pDTG1 (Dennis and Zylstra, 2004) and NAH7 (Sota et al., 2006) have shown extensive homology in their replication, partitioning and transfer loci (Sevastsyanovich et al., 2008). The latter study has phylogenetically analyzed 30 IncP-9 plasmids, and based on

phylogenetic analysis of *rep* and *oriV* sequences revealed nine ($\alpha, \beta, \gamma, \delta, \varepsilon, \eta, \zeta, \theta$) IncP-9 subgroups with 7-35 % divergence between them.

7. Plasmid host range

Plasmids are divided into two groups, based on the range of bacteria that can receive and maintain the plasmid: a narrow-host range group (NRH) and a broad-host range (BHR), the latter frequently called “promiscuous plasmids”. The first division into these two groups was made in 1972 by Datta and Hedges (1972), who defined BHR plasmids as those able to transfer among *Enterebactecteria sp.* and *Pseudomonas sp.* However, since then the definitions have been readjusted and currently it is suggested that BHR plasmids transfer and maintain among bacteria belonging to different phylogenetic subgroups (Top et al., 1998b).

Researchers have suggested that one of the main differences between BHR and NHR plasmids could be due to their replication systems., e.g. some studies reported a reduction of plasmid host range for *trfA* (replication initiator protein) deficient IncP-1 plasmid (Smith and Thomas, 1987).

Moreover, based on comparative analyses it was revealed that BHR plasmids have a lower number of restriction sites than NHR plasmids, increasing their chance to escape an initial contact with the restriction system of the recipient bacteria (Meyer et al., 1977) (see below). De Gelder et al. observed that a single point mutation in a TrbC protein (putative prepilin, see above) of a broad-host range pB10 plasmid, might result in plasmid’s host range expansion (De Gelder et al., 2008)

Interestingly, the same author revealed that host range of a pB10 plasmid within an activated-sludge microbial community was significantly influenced by the type of donor strain (Figure 5) (De Gelder et al., 2005). The latter study indicates the need of diverse novel strategies, for a better understanding of the spectrum of hosts to which a conjugative plasmid transfers in a microbial community, as attempted in Section 1.

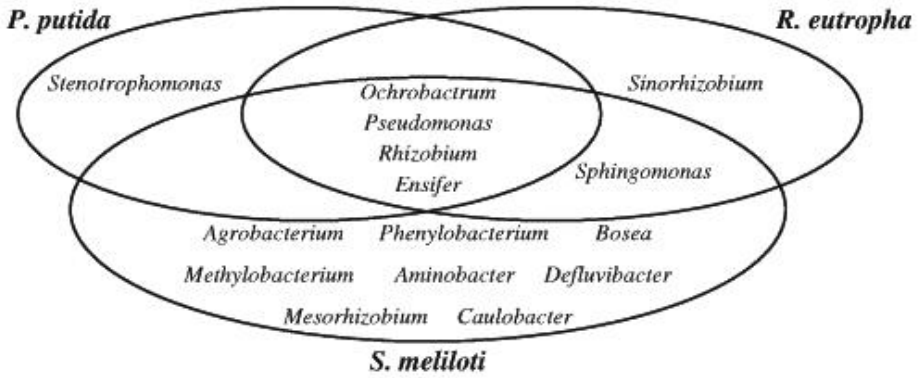

Figure 5. The phylogenetic diversity of transconjugants in the activated sludge obtained in the separate matings with pB10 (IncP-1) plasmid harboring donor strains *Pseudomona putida, Ralstonia eutropha* and *Sinorizobium meliloti* (De Gelder et al., 2005)

7.1 Host range of IncP-1 and IncP-9 plasmids

IncP-1 plasmids have previously been shown to have a broad host range, transferring between most of the tested Gram-negative species (Dröge et al., 1999; Geisenberger et al., 1999), even to strains where the RP4 plasmid could not be replicated (Guiney and Yakobson, 1983). Among the first reported transfers of IncP-1 between Gram-negative and Gram-positive bacteria was made by Breton et al. (Breton et al., 1985), which revealed transfer and chromosomal integration of the RP4 plasmid.

The exceptionally broad host range capability of IncP-1 transfer genes has been revealed since by numerous studies. For instance, mobilization of a non-self transmissible plasmid (RSF1010) (IncQ) by chromosomally-integrated RP4-*tra* genes was demonstrated between *E. coli* and Gram-positive *Streptomyces lividansand* and *Mycobacterium smegmatis* (Gormley and Davies, 1991). Moreover, mobilization of a shuttle plasmid pECM1 from *E. coli* to *Corynebacterium glutamicum* (Gram-positive bacteria) was performed by a plasmid with integrated RP4-*oriT,* double *E. coli* (*ori*V_E) and *C. glutamicum* (*ori*V_C) origins of replications and chromosomally inserted RP4 *Tra* genes (Schafer et al., 1990). Adding an additional origin of replication unique for Gram-positive bacteria Schafer et al. increases the possibility of the shuttle plasmid's maintenance in a new host, as the maintenance requisites plasmid

replication. These observations suggest that a generally assumed natural border for plasmid exchange, i.e. Gram barrier, does not rely on differences in cell well structure among Gram-negative and Gram-positive bacteria, as proposed by numerous of studies.

pWWO (TOL) plasmid seems primarily to transfer among *Pseudomonas sp.*. Although the TOL plasmid has also shown transfer to *Erwinia sp. Escherichia coli* (Ramos-Gonzalez et al., 1991), *Rhizobium sp.* (Jussila et al., 2007), *Serratia ficaria* and *Hydrogenophaga palleronii* (Nancharaiah et al., 2003), it is still considered as a narrow-host-range plasmid by most scientists.

7.2 Retrotransfer and retromobilization

Some conjugative plasmids can also facilitate the movement of (mobile) genetic elements from recipient to donor bacteria, i.e. in the opposite direction to conjugal plasmid transfer, by a process of retrotransfer or retromobilization. This could potentially increase the chance of a MGE to expand its host range. The mechanisms of this process are still unknown, but it has been shown that for the RP4 plasmid, the plasmid must be transferred into the recipient cell before it can mobilize a plasmid back to the donor (Sia et al., 1996). To date, potencial for retrotransfer and retromobilization has been demonstrated for plasmids belonging to the IncP-1 (Mergeay et al., 1987; Top et al., 1995), IncP-9 (Ramos-Gonzalez et al., 1994), IncF (Heinemann and Ankenbauer, 1993) and few other Inc groups.

7.3 Restriction–modification system as a transfer barrier

It has been suggested that one of the greatest barriers for plasmid establishment and persistence among distantly-related bacteria is indisputably the bacterial restriction–modification system. The first site-specific restriction endonuclease (HindII), isolated from *Haemophilus influenzae* in 1968 by Smith H.O. and Wilcox K.V. (Smith and Wilcox, 1970), cleaves foreign *ds*DNA at a six-base specific sequence, while all currently known restriction-endonucleases recognize a 4bp (e.g. Sau3AI) to 8bp (e.g. NotI) sequence. However, an incoming ssDNA will not immediately be a target for endonucleases, which gives ssDNA the possibility for quick incorporation into the cell chromosome(Cohan, 2002). This is probably the reason why, e.g. Bertram *et al.* (1991), used the chromosomal integration of a conjugative transposon Tn916

while studying transfer from diverse Gram-positive to Gram-negative bacteria, and vice versa.

8. Examination of plasmid transfer

8.1 Limitations and advantages of vitro and in situ studies

Estimation of plasmid transfer and host range has traditionally been done via two main approaches: 1.) the plasmid transfer from a donor strain to pre-selected recipient bacteria under controlled in vitro conditions (e.g. filter matings) and 2.) transfer from an introduced donor strain to an indigenous community in an in situ test system.

As a consequence of still-unknown physiological and growth requirements for the majority of indigenous bacteria, a minor fraction of indigenous bacteria is suggested to be culturable, ranging from 0.01-5 % (Torsvik et al., 1990; Amann et al., 1995; Sait et al., 2002). Therefore, the first experimental approach will be exceptionally limited to a small fraction of pre-selected bacteria. The latter approach will, besides some advantages described below, still frequently require cultivation of indigenous transconjugants on selective media, prior to their appropriate characterization. Therefore, development of novel cultivation-minimal or cultivation-independent methods for examination of bacterial processes is a necessity.

A few studies have revealed that bacterial culturability decreases as a function of soil depth, suggesting a substrate–accelerated death or a large appearance of viable but non-culturable (VBNC) state among oligotrophic bacteria exposed to high-substrate commercial media (Byrd et al., 1991; Sait et al., 2002). As physical isolation and cultivation of indigenous bacteria is still fundamental for bacterial physiological, biological, chemical, and nutritional characterization, a number of studies have tried to deal with improved cultivation of uncultivable bacteria by, e.g., creation of environmentally-representative media (e.g. soil extract) (Shimomura et al., 2006). Other studies, using a flow-cytometric sorting of individual encapsulate cells (Zengler et al., 2002) or a continuous long-term incubation of microcolonies on membranes on low substrate media (Rasmussen et al., 2008) have succeeded in growing previously-unculturable organisms in pure cultures. Others have used more complex systems e.g. SSMS (soil substrate membrane system) to enrich for slow-growing oligophiles, allowing their easier isolation (Ferrari and Gillings, 2009).

The *in situ* microcosms have emerged as a result of low bacterial culturability and technical challenges linked to experiments in the field (Dröge et al., 1999). Microcosms are designed to mimic original conditions (i.e. ecosystem) (Daane et al., 1997; Hill and Top, 1998) and permit researchers to manipulate various parameters. However, these should still be considered as enclosed systems and need to be scaled up for a trustworthy validation of previously-obtained results (Hill and Top, 1998). To minimize the "*closed system*" bias, more complex systems have been constructed. Sun et al. (1999) constructed a vertical soil column with percolation of nutrients and a continuous-flow column reactor with glass beads, while Pearce et al. (2000) made a rhizosphere model system with hollow fibre membranes simulating a root surface. A glass-beads microcosm system with environmentally relevant oligothropic conditions was applied in our study (Appendix 2).

8.2. Examination of plasmid transfer by novel reporter gene methods

In parallel with improvement of cultivation media, many researchers have focused on the development of various cultivation-independent methods, while investigating diverse bacterial processes, e.g. diversity, gene copy number, gene transfer etc. (Amann et al., 1995; Sørensen et al., 2003; Fey et al., 2004). Among these, a particularly interesting approach for the current thesis is a reporter gene system designed by Christensen et al. (1996), which allows bacterial visualization and tracing of MGE (e.g. plasmid) transfer among bacteria due to expression of a green fluorescent protein (GFP) (Chalfie et al., 1994). The approach was later optimized (Christensen et al., 1998b; Dahlberg et al., 1998) by merging *gfp* gene downstream from a synthetic *LacZ* (*LacI*-repressible) promoter. Upon chromosomal insertion of a *LacI* gene the *gfp* expression will be repressed in a plasmid donor cell, but will be restored upon transfer to the indigenous recipient cells (Figure 6).

In parallel with *gfp*-based marker system, other systems using zygotic induction of reporter genes, e.g. a red fluorescent protein (DsRed) have been developed (Tolker-Nielsen and Molin, 2000), and are frequently used in combination with a *gfp*-system for differentiation between plasmid donor and recipient cells, as presented in Figure 6.

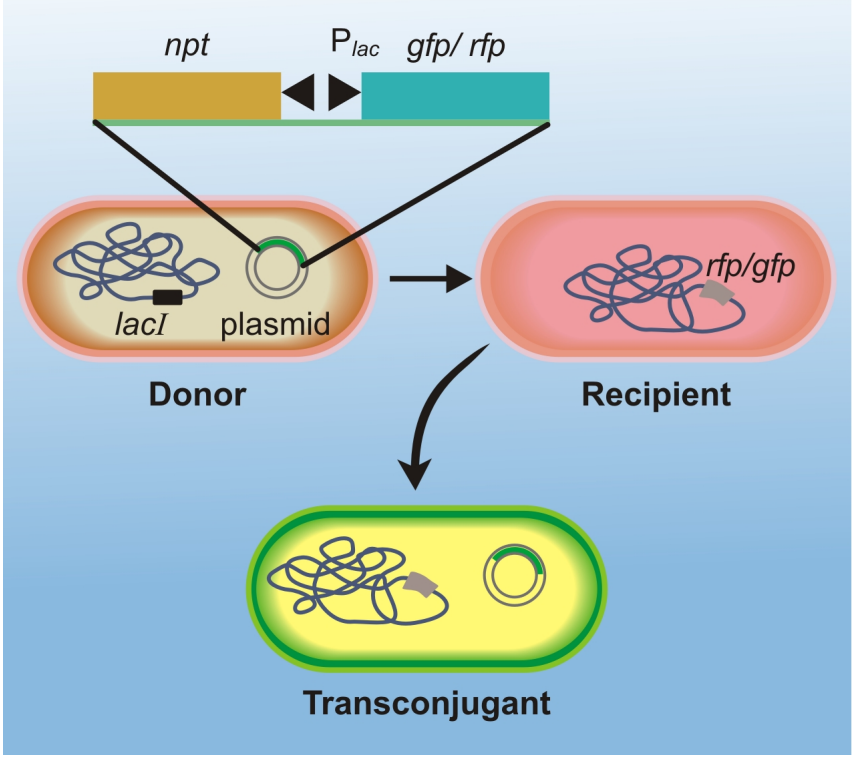

Figure 6. A schematic of the reporter gene merged downstream from a synthetic LacZ promoter. The expression of the plasmid encoded reporter genes *(e.g. gfp)* is repressed in the donor cell by chromosomally tagged *lac*I gene. Upon the plasmid transfer to a recipient cell that does not carry *lac*I, but e.g. constitutively expressed a red fluorescence gene (*rfp*), the plasmid encoded *gfp* gene will be expressed and the transconjugant cell can be visualized by its fluorescence (yellow). *npt* kanamycin resistance gene. Illustration made at DTU-Environment, Technical University of Denmark.

The approaches relying on the above type of plasmids marked with fluorescent reporter genes (e.g. *gfp*) are frequently used in combination with confocal laser scanning microscopy and flow cytometry for *in situ* visualization and quantification of plasmid transfer among bacteria in biofilms, activated sludge, phylloplane, the rhizosphere, etc. (Christensen et al., 1998a; Normander et al., 1998; Geisenberger et al., 1999; Hausner and Wuertz, 1999; Musovic et al., 2006), as well as for host range determination in the rhizosphere (Musovic et al., 2006). This approach allows detection of plasmid transfer to indigenous organisms with still-unknown phenotypes. Nevertheless, a minor limitation of

"*LacZ promoter-reporter gene*" system needs to be addressed. For instance, due to the presence of a *LacI* repressor gene in most enteric bacteria, transfer to these bacteria will mainly not be detected by this construct, indicating the unsuitability of this system for experiments performed in, e.g., intestinal gut flora or freshly manured agricultural soils, as the generally low content of enteric bacteria in soil (Ishii et al., 2006) could transiently be changed upon manure application (Jiang et al., 2002).

These cultivation-independent approaches have, however, shown their superiority to traditional methods. Hausner and Wuertz (1999) used reporter-gene approach and revealed that transfer frequency of pRK415::*gfp* plasmid in a biofilm was a three orders of magnitude higher using this approach, than inferred by cultivation methods. Interestingly, Sørensen et al. (2003) have observed that even a simple cell quantification of laboratory *E. coli* or *P. putida* strains will be underestimated when enumerated on solid media in comparison to a flow-cytometric approach. This suggests that reduced colony formation might not only result from unknown bacterial requirements, but might also rely on gene-expression level, i.e. a continuous expression of catabolic and resistance encoding genes. Consequently, in the present thesis, a combination of soil extract mimicking the environment, the reporter gene system, advanced microscopy and micromanipulation was applied to estimate the fraction and diversity of bacteria in a microbial community that were able to receive a conjugal plasmid.

8.3. Expressing plasmid transfer incidences

In reviewing the microbiological literature, a complete lack of any standard approach for expressing plasmid transfer incidences is revealed. The plasmid transfer is expressed as a transfer frequency by most microbiologists, and is often estimated as the number of enumerated transconjugants per number of donors (T/D) (Fernandez-Astorga et al., 1992; Muela et al., 1994), per number of recipients (T/R) (Schwaner and Kroer, 2001) or per number of donor and recipient cells $(T/(D \times R)^{1/2}$ (Sengelov et al., 2001). Furthermore, a transconjugant ration (TR) used by Boon et al (2006) was estimated a the amount of transconjugants per total amount of donors, recipients and transconjugants in a sample. However, these measures will not truly allow authors to distinguish between an actual donor-to-recipient plasmid transfer incidence and subsequent

growth of transconjugants, and will furthermore vary with parental cell density, duration of mating, etc.

Rates of plasmid transfer can, however, also be expressed as the intrinsic kinetic coefficient, *k*t1 (Levin et al., 1979), which is a more accurate way of expressing plasmid transfer. It should ideally be independent of parental cell density and time, as it describes the probability that transfer take place after a donor-recipient cell collision (Levin et al., 1979; Simonsen et al., 1990). In this form, transconjugant appearance is directly proportional to the product of average donor and recipient concentrations over a given time period. The constant of proportionality is called the plasmid transfer rate constant. However, very few plasmid transfer studies with *k*t1 values are available in soils. Sudarshana and Knudsen (1995) have estimated plasmid transfer rate constants (γ = transconjugants x (donors x recipients)$^{-1}$ x h^{-1}) in plasmid transfer study in the pea spermosphere and rhizosphere. *k*t1 was also used by MacDonald et al. (1992). and Smets et al. (1993) in studies examining the effects of substrate concentration on plasmid transfer rate.

9. Factors controlling conjugation

The extent of plasmid exchange among bacterial communities is affected not only by the type of host organism (De Gelder et al., 2005), bacterial relatedness or plasmid nature (BHR vs. NHR) discussed above, but also affected by various other parameters, both abiotic (e.g. temperature, moisture, pH, particles) and biotic (e.g. plants, animals etc). (Khalil and Gealt, 1987; Richaume et al., 1989; Fernandez-Astorga et al., 1992).

In general, previous studies have revealed no significant effect on plasmid transfer in *E. coli* strains was observed upon changing pH values from 5.0-8.5 (Fernandez-Astorga et al., 1992), while ca. 20% soil moisture (Richaume et al., 1989) and larger fraction of clay particles in a soil microcosm (Pukall et al., 1996) had a positive effect on plasmid transfer.

Human-made changes in the environment, e.g. treatments of agricultural soils by manure (addition of microbes and nutrients), tillage, etc., can temporarily or permanently change the indigenous bacterial community structure in a way that these become more or less permissive toward incoming bacteria or their MGEs. Although different plasmid transfer studies have been made in different environments, these have still been unable to eliminate e.g. transconjugant outgrowth (Geisenberger et al., 1999; Hausner and Wuertz, 1999).

Therefore, the actual part of an indigenous microbial community that actively engages in uptake and exchange of plasmid is still not measured. Linking community structure to the estimate of that community's permisivity toward a certain plasmid type will be crucial, while investigating for instance the potential effects of application of manure from antibiotic-treated animals, as well as prior introduction of genetically modified organisms for bioaugmentation purposes.

Q1. Can we estimate the true fraction of an indigenous microbial community, which can receive and maintain a mobile genetic element?

Plasmid transfer in soil is mainly associated with surfaces characterized by larger bacterial density, such as e.g. clay particle sites for bacterial adsorbtion (van

Elsas et al., 1988) or the presence of various hotspots (e.g. rhizosphere, spermosphere). Compared to the more oligotrophic bulk soil, the pea and barley rhizosphere (soil around the plant root influenced by root exudates and sloughed cells) was revealed by Schwaner.& Kroer to lead to higher plasmid transfer among bacteria (Schwaner and Kroer, 2001).. On the contrary, Sudarshana et al. (1995) reported no significant influence of pea spermosphere (soil-seed interfaces) on plasmid transfer rate constants (inferred as transconjugants x (donors x recipients)$^{-1}$ h^{-1})) in comparison to bulk soil.

In view of the fact that soil can be considered as a system with more (bulk soil) or less (hotspot) apparent oligothropic features, it is consequently very important to examine the effects of generally present oligotrophic conditions on the exchange rate of mobile genetic elements.

Q2. Is horizontal gene transfer, i.e. conjugation, relevant under environmentally oligotrophic conditions ?

Selective pressure is another environmental factor frequently associated with an increased occurrence of resistance-encoding genes (e.g. *merA*) at polluted sites (Khesin and Karasyova, 1984; Barkay, 1987; Smalla et al., 2006). Increased abundance of resistance genes in a bacterial community might, however, be the result of resistant bacteria outgrowth and/or an increased propagation of functional resistance genes carried on MGEs. A high abundance of e.g., IncP-1*beta* plasmids was recently reported at highly mercury-contaminated sites (Smalla *et al.*, 2006). Smit et al. (1998) reported on a ca. 10-fold higher isolation frequency of mercury-resistance plasmids when the filter matings with a well-defined recipient strain contained wheat rhizosphere soil suspensions from microcosms initially amended with high levels of mercury. As the mercury amendments resulted in a >10^3-fold decrease in total cell number, the author's observation is most likely the result of an advantageous growth of a few resistant bacteria in the microcosm, prior to the soil suspension matings with recipient strains on a filter. However, the presented approach can be used to increase the chance of isolation of MGEs with certain phenotypes. A few other in vitro studies have actually shown that the presence of selective pressure (i.e.

kanamycin and mercury) can have a negative effect on conjugative transfer of TOL and pRO103 plasmids, which encode resistance to those compounds, respectively (Boon et al., 2006; Johnsen and Kroer, 2007). Therefore, it is very important to investigate the effect of selective pressure on dissemination of MGEs (e.g. plasmids) encoding respective resistance under environmentally relevant conditions.

Q3. Can the presence of inhibitory agents (at concentrations below where they affect cell growth) stimulate the dissemination of mobile genetic elements which encode for resistance against them under environmentally oligotrophic conditions ?

The importance of the metabolic activity of bacterial cells on the exchange of mobile genetic elements (e.g. plasmids) has been addressed in different studies. It is generally accepted that an increased metabolic state or nutrient availability to plasmid donor and recipient cells is positively linked to an increased plasmid transfer (Muela et al., 1994; Fox et al., 2008). Although the physiological status of both donor and recipient cells in a conjugal process is important, some scientists have suggested that the physiological status of donor cell is of critical importance (Sudarshana and Knudsen, 1995).

However, Pearce et al. (2000), using a rhizosphere model system, reported a general increase in the number of transconjugants as a function of increasing substrate (glucose) concentration, but plasmid transfer frequencies (inferred as transconjugant per donor) decreased exponentially with increasing substrate concentration. Interestingly, Hausner and Wuertz (1999), using a direct microscopic visualization of RP4:*gfp* plasmid in a biofilm, revealed no significant correlation between plasmid transfer and nutrient concentration. Moreover, other studies have revealed a similar absence of correlation between plasmid transfer frequency and nutrient availability, but only when the nutrient concentrations in their systems were above a certain threshold value, as positive correlations were observed at lower tested concentrations (Normander et al., 1998; Johnsen and Kroer, 2007).. Starvation of plasmid donor cells for 24 hours prior to experiments was shown to significantly reduce plasmid transfer frequency (Johnsen and Kroer, 2007). Using novel methods, e.g. an unstable

reporter gene only expressed and visualized in continuously active cells, it was recently reported that transfer occurs exclusively to metabolically active recipient cells (Haagensen et al., 2002). A microscopic technique recently developed by Walczysco (Walczysko et al., 2008) allows scientists to determine the physiological status of a single cell *in situ* without disturbing a system, by measuring the RNA:DNA ratio by a fluorescence lifetime imaging technique. Therefore, the possibility to link a particular bacterial physiological status with plasmid transfer *in situ* has arisen, and it is important to investigate:

Q4. Can we predict the plasmid transfer rate, based on the physiological status of plasmid donor?

The most of above reviewed studies have estimated the effect of various controlling factors on conjugation by the population averaged measurements, assuming e.g. optimal cell-to-cell contact conditions, a full transfer proficiency of each donor cell, and minimizing the effect of population's heterogeneity. Therefore, using the novel approaches described above, it is essential to estimate:

Q5. What is the transfer proficient fraction of a plasmid donor population under optimized surface-mating conditions?

10. Conclusion and perspectives

The introduction of inanimate contaminants such as pesticides, heavy metals, and antimicrobial drug residues or animate contaminants such as intestinal bacteria, via industrial and agricultural practices, can greatly affect the composition of the indigenous microbial community in the receiving environment. A typical observation has been an increased fraction of bacteria carrying resistance- or degradative genes after such events. Often these functional traits are encoded by plasmids, which are present in the exposed environments; and whose quick spread among bacteria within the community could increase the potential for a useful response such as biodegradation or bioresistance. On the other hand, the introduction of multiple resistance plasmids by exogenous bacteria could create a large reservoir of resistant gene carrying bacteria -if spread to the indigenous community occurred- with potential broad consequences for public health. Therefore, understanding the determining forces and quantifying the actual extent of horizontal gene exchange in the environment is deemed essential in order to predict the potential benefits and threats associated with the release and spread of mobile genetic elements carrying functional genes.

This thesis focused on the dissemination capacity of conjugative plasmids carrying biodegradation or resistance encoding genes. A broad array of experimental approaches were optimized or newly developed in order to examine the effects of various controlling factors on plasmid dissemination under environmentally relevant conditions.

The main questions to be addressed and conclusions drawn from this thesis are:

Q1. *What is the true fraction of an indigenous microbial community, which can receive and maintain a mobile genetic element?*

In attempt to answer the question, I have developed a new approach to quantify the true fraction of a microbial community partaking in plasmid exchange. This method merges reporter gene technology with advanced microscopy and a cultivation-minimal incubation. An exceptionally high transfer incidence of the RP4:*gfp* (IncP-1) plasmid from *Pseudomonas putida* to bacteria in soil cell suspension (up to 1 transfer per 2000 soil bacteria) was observed, suggesting a quick spread and persistence on IncP-1 antibiotic resistance plasmids to indigenous soil bacteria. In addition, the

new method revealed a wider phylogenetic range of plasmid recipients than was observed by traditional selective culturing based methods.

Q2 & Q3. *Is horizontal gene transfer relevant under environmental conditions, and can the presence of inhibitory agents stimulate the dissemination of mobile genetic elements that encode for their resistance* ?

Using a cultivation-independent quantitative-PCR method, I revealed that dissemination of a Hg-resistance (*merA*) encoding IncP-1 plasmid (pKris-1) from an *E. coli* strain to a synthetic bacterial community occurred under oligotrophic conditions. In addition, I demonstrated that the transfer rate of pKris-1 was greatly enhanced (up to 1.000 fold) in presence of Hg at 1 μM level (compared to 0 or 0,1 μM Hg). This larger plasmid dissemination allowed the community to successfully cope with a subsequent 10 μM Hg (compared to 0 or 0,1 μM Hg). The results linking the level of selective pressure and dissemination of plasmid with specific resistance gene might ultimately allow the prediction of plasmid behavior in natural environments and the potential for bioaugmention by gene transfer.

Q4. *Can we predict the plasmid transfer rate based on the physiological status of plasmid donor?*

I investigated the effect of physiological status of a TOL (IncP-9) plasmid carrying *P. putida* KT2442 strain on transfer rate to two *Pseudomonas* recipient strains. Within the range examined (μ_{max} = 0,071 to 0,2128), specific growth rates of donor cells, had very little impact on the measured plasmid transfer kinetic rates. The same trend was observed when transconjugants were measured by selective plating or by flow cytometric approach using fluorescent reporter genes. However, the transfer rates were 1000 times higher when inferred with the last approach. I speculate that bacterial physiological status above a certain threshold-value will have a limited effect on the plasmid transfer, allowing an easier, less nuanced prediction of bacterial physiological status and plasmid transfer rate.

Q5. *What is the transfer proficient fraction of a plasmid donor population*?

The transfer proficient fraction of exponentionally pregrown *P. putida* KT2442 (TOL:*gfp*) cells in a surface mating constructed to optimize cell-to-

cell contact with recipient cells, was revealed to be quite low (ca. 8 %).The fraction was further reduced (ca. 100 times) under stirred liquid conditions. The observation that a large fraction of a plasmid donor population, even in a healthy physiological status, will not act as plasmid donors is novel, and needs to be considered while predicting the donor cells behavior in experiments or expressing plasmid transfer frequencies.

The novel cultivation-minimal approach to estimate plasmid permissive fractions, can be now applied to examine dissemination and mobilization capacity of various degradation- or resistance encoding plasmids belonging to diverse incompatibility groups. Bacterial communities from different environments or exposed to different treatments can be tested. Estimating the dissemination capacity of plasmids that are typically found in Gram-positive bacteria is of particular interest, as these plasmids are under examined. Furthermore, estimating the host range for relevant plasmids, harbored in diverse host strains (Top et al.), combined with micro-manipulative isolation is an important step in characterization of core members of indigenous communities.

The finding that dissemination of the *merA*-encoding plasmid was enhanced in response to selective pressures is important. However, the effect of selective pressure on dissemination of other plasmids not carrying resistance genes via conjugation or mobilization also needs to be considered prior to a possible bioaugmentation process.

Using modern approaches (reporter gene, microscopy etc) I succeeded in estimating true plasmid transfer rates, unbiased by subsequent outgrowth of transconjugants. I also revealed that, for our model system, only one in every ten cells in a plasmid donor population contributes to plasmid disseminations. Hence, it will be interest to examine how this transfer proficient donor fraction may vary as a function of physiological and environmental conditions.

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12. Appendix

I. **Sanin Musovic**, Arnaud Dechesne, Jan Sørensen & Barth F. Smets. A novel assay to measure the permissivity of microbial communities towards horizontal receipt of exogenous mobile elements. *Submitted manuscript: Applied and Environmental Microbiology (AEM02713-09).*

II. **Sanin Musovic** and Barth F. Smets. The toxic effect of elevated Hg doses is alleviated by *in situ* transfer of the Hg resistance plasmid, pKris-1, to a synthetic microbial community. *Manuscript.*

III. **Sanin Musovic**, Charles Halouze, Claus Sternberg and Barth F. Smets. The effect of physiological status of Pseudomonas putida KT2442 on transfer rate and proficiency of a conjugal TOL plasmid. *Submitted manuscript: FEMS Microbiology Letters (FEMSLE-10-01-0074).*

The papers are not included in this www-version but can be obtained from the library at DTU Environment. Contact info: Library, Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2800 Kgs. Lyngby, Denmark or library@env.dtu.dk.

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